REMARKS/ARGUMENTS

Claims 10, 12-14, 16-21 and 36-43 have been examined and are currently pending in the above-identified application. Claims 40 through 43 have been canceled without prejudice to prosecution of the subject matter encompassed by the claims in a related copending application. Claim 10 has been amended to further clarify the present invention. The amendment of claim 10 does not add new matter and is further discussed below. Applicants note that the prior rejection of claim 36 under 35 U.S.C. §112, first paragraph has been withdrawn. Applicants respectfully request reconsideration of the application in light of the following remarks.

Rejections Under 35 U.S.C. §103(a)

Claims 10, 12-14, 16-21, and 36-43 remain rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,788,963 (1998) in view of Thurnher *et al.* (1997) and Ramoner *et al.* (1998).

The Examiner again states that the '963 patent teaches a method for producing an anti-tumor cell, antigen specific cytotoxic T cell (CTL) response comprising administering to a patient an effective amount of human DCs, the DCs having been exposed *in vitro* to the prostate tumor associated antigenic fragment PSM-P1 (SEQ ID NO:1) derived from various sources including tumor cell lysates and purified antigens. The reference has also been cited by the Examiner as further teaching that the DCs are obtained from peripheral blood, have been cryopreserved, have been obtained from a healthy HLA matched donor, are treated to provide an extended life span, and can be administered to a metastatic prostate cancer patient.

Thurnher *et al.* has been again cited by the Examiner as teaching the *in vitro* maturation and activation of DCs with BCG. The reference is also believed to further teach the DCs matured in the presence of BCG may also take up tumor antigens and thus, then be capable of activating tumor-reactive T cells in a cytokine milieu that favors the generation of a strong

anti-tumor CTL response. The reference conclusion is also cited by the Examiner as teaching tumor-antigen loading of DCs cultured in BCG.

Ramoner *et al.* is believed by the Examiner to further extend the work of Thurnher *et al.* In particular, the Examiner believes that Ramoner *et al.* teaches that BCG "is a potent activator of human DCs." The reference is also cited as teaching that BCG stimulates the ability of DCs to activate T cells and that BCG could be used in DC based tumor immunotherapy.

Therefore, the Examiner believes that it would have been *prima facie* obvious to one of ordinary skill in the art at the time the invention was made to perform a method for producing an anti-tumor cell, antigen specific CTL response comprising administering to a patient an effective amount of human DCs, said DCs having been exposed *in vitro* to a tumor antigen. In addition, the DCs used in these methods could have been obtained from peripheral blood, cryopreserved, or they could have been obtained from a healthy HLA matched donor, prepared for an extended life span, and they could have been administered to a metastatic prostate cancer patient, as taught by the '963 patient. The Examiner also believes that one of ordinary skill in the art would have been motivated to add BCG to the *in vitro* exposure of DCs to antigen for an improved anti-tumor, antigen specific CTL response, given the combined teachings of Thurnher *et al.* and Ramoner *et al.* that: 1) BCG causes the maturation of DC and thus, the DCs are then capable of activating tumor-reactive T cells in a cytokine milieu that favors the generation of a strong anti-tumor CTL response and 2) BCG "is a potent activator of human DCs", BCG stimulates the ability of DCs to activate T cells, and BCG could be used in DC based tumor immunotherapy. Further, the rejection of claims 36 and 40 has been maintained because the Examiner believes that the claims encompass only routine optimization of the claimed method and therefore, fall well within the purview of one of ordinary skill in the art at the time of the invention has also been maintained.

It remains the Examiner's position that the improved anti-tumor, antigen-specific CTL response obtainable with the combined method establishes proper motivation to combine

the references. Further, it appears that the Examiner is convinced that Applicant has merely provided characterization of how the improved immune response is achieved, *i.e.*, through enhanced MHC Class I presentation. In support of this position, the Examiner states that the reference teaches that *in vitro* culture of DCs with BCG, among other things, enhances expression of both CD83 and CD86 expression (DC maturation markers). The Examiner also asserts that expression of CD86, in particular, comprises a costimulatory molecule that would enhance the CTL response and that for this reason alone it would be obvious to combine the methods of the '963 patent and Thurnher *et al.*

Applicants must again respectfully disagree with the Examiner's argument that motivation for the combination of the "963 patent and Thurnher *et al.* is present. The present invention is directed to a method for producing an antigen specific cytotoxic T cell response, comprising: administering, to a patient in need thereof, a composition consisting essentially of an effective amount of human dendritic cells, exposed *in vitro* to a soluble, exogenous tissue specific antigen and bacillus Calmette Guerin (BCG) or BCG with lipopolysaccharide (LPS) to induce antigen processing and to promote Major Histocompatibility Complex- (MHC-) class I presentation of the antigen, such that after administration the human dendritic cells presenting the antigen in the context of MHC-class I elicit the antigen specific cytotoxic T cell immune response. The compositions of the present invention consist of essentially only an effective amount of the human dendritic cells presenting the antigen in the context of MHC-class I as the active ingredient, and does not include BCG. Claim 10 has been amended to recite "a composition consisting essentially of an effective amount of human dendritic cells, exposed *in vitro* to a soluble, exogenous tissue specific antigen and bacillus Calmette Guerin" to further clarify and more definitively recite the present invention. As such, claims 10, 12-14, 16-21, and 36-43 are not unpatentable over U.S. Patent No. 5,788,963 ("the '963 patent) in view of Thurnher *et al.* (1997) and Ramoner *et al.* (1998).

Previously, Applicants have argued that Thurnher *et al* do not disclose that the *in vitro* exposure of DCs to BCG provides a cytokine milieu that favors the generation of a strong

anti-tumor CTL response. Applicants believe that the passage referred to by the Examiner addresses a cytokine milieu that is induced *in vivo* upon the administration of live BCG, where the anti-tumor effect of BCG *in vivo* therapy is postulated to "result from the uptake of tumor antigens along with BCG organisms as a consequence of the intra- or peri-tumoral administration of BCG." Thurnher *et al.* further speculate that "the presentation of tumor antigens by DCs that have been fully activated by BCG will favour the activation of tumor-reactive T-cells" and that "the concurrent activation of mycobacteria-reactive T-cells (even by the same DC) may create a cytokine milieu which further facilitates activation of tumor-reactive T-cells and enables the generation of a strong-anti-tumor T-cell response." The fundamental difference between Thurnher *et al.* and the present invention resides in the difference between *in vivo* administration of live BCG and the administration of dendritic cells following prior, *in vitro*, pulsing of the dendritic cells with BCG. There is nothing in the speculations of Thurnher *et al.* that would lead the skilled artisan to reason that pulsing immature dendritic cells with BCG and tumor associated antigen *in vitro* would provide a mature dendritic cells that present the antigen in the context of MHC-class I and that are capable of eliciting an antigen specific cytotoxic $CD8^+$ T cell immune response upon subsequent administration to a patient.

Thurnher *et al.* disclose i) BCG induces the maturation of immature dendritic cells as demonstrated by the induction of the expression of CD83, the up-regulation of CD86 and the down-modulation of the endocytosis mechanisms responsible for FITC-dextran uptake, ii) BCG induce the aggregation of dendritic cells, iii) BCG stimulates TNF-α protein release by dendritic cells. None of the indicated dendritic cell characteristics would convince the skilled artisan that BCG would induce the up-regulation of MHC class-I expression or the upregulation of antigen presentation in the context of MHC class I, or that the mature dendritic cells produced by Thurnher *et al.* could induce a class I-restricted, tumor-antigen specific cytotoxic $CD8^+$ T cell response either *in vivo* or *in vitro* as set forth in the present invention. In fact, a prior publication authored by Pithie *et al.* (*Thorax* 47:695-701, 1992, copy attached hereto) discloses that vaccination of individuals with live BCG induces $CD4^+$ T cells that can lyse autologous macrophages presenting mycobacterial antigen.. See conclusion of Abstract on page 695, left

column. Further, Lang *et al.* (*J. Immunol.* 168:3786-3792, 2002, copy attached hereto) in a post filing article report that the *in vivo* blockade of CD86 by a monoclonal antibody specific for the receptor inhibits $CD4^+$ T cell activation. These data suggest that the increase in CD86 demonstrated by Thurnher *et al.* would likely result in the up-regulation of a $CD4^+$ T cell response as disclosed by Pithie *et al.*, as opposed to a class I-restricted $CD8^+$ T cell response. Therefore, even subsequent to the filing date of the present invention the skilled artisan would not have a reasonable expectation that pulsing immature dendritic cells with BCG and a selected antigen would result in the induction of an antigen specific cytotoxic $CD8^+$ T cell response. As such there is no motivation to combine the '963 patent with Thurnher *et al.*

The Examiner has also asserted that the skilled artisan would be motivated to combine the methods of Thurnher *et al.* and Ramoner *et al.* with the '963 patent because of the teachings that BCG can be used in tumor immunotherapy. Applicants do not agree with the Examiner's summary of the teachings of Thurnher *et al.* or Ramoner *et al.*, nor the conclusion reached by the Examiner. Thurnher *et al.* teach in the discussion that "[t]he effectiveness of BCG therapy may be increased by *ex vivo* tumor antigen-loading and DC activation. In principle, this could be achieved by the use of cultured DCs, which can be pulsed with tumor-derived antigens *in vitro* and stimulated with BCG prior to re-infusion." This suggestion of the use of BCG-matured DCs is in the context of increasing the effectiveness of BCG-based (rather than tumor-derived) therapy, *i.e.*, in the context of instilling live BCG directly into the bladder to cure superficial bladder cancer, and as such one of skill in the art would treat a patient with both the administration of live BCG as well as DCs matured *in vitro* with BCG. The present invention does not include the administration of live BCG either separately or as an element of a composition comprising BCG matured DCs, which Applicants believe clearly distinguishes the invention from what may be disclosed or suggested by Thurnher *et al.* Further, as above, the disclosure of Thurnher *et al.* does not disclose or suggest that the BCG pulsed DCs up-regulate MHC class I or can induce an antigen specific cytotoxic T cell response.

Live BCG therapy of cancer at the time of the present invention , as well as today, is limited to superficial bladder cancer. The anticipated increase effectiveness of live BCG therapy as suggested by Thurnher *et al.* thus applies to bladder cancer, where the tumor is treated by the instillation of live BCG directly into the bladder. This application would therefore not extend to a disease, such as prostate cancer or to other cancers that are not treated with live BCG.

Ramoner *et al.* does not provide any teaching that would further support the Examiner's assertions. As in Thurnher *et al.*, Ramoner *et al.* demonstrate that BCG stimulation of immature dendritic cells induces the release of IL-8 and that BCG stimulates allostimulatory potential of human dendritic cells as measured by a mixed lymphocyte reaction (MLR). Further, Ramoner *et al.* conclude merely that BCG is a potent activator of human dendritic cells, that it stimulates IL-8 production as well as the ability of dendritic cells to activate T cells. Therefore, the teachings of Ramoner *et al.* further confirm that live BCG would likely induce a class II response and the teachings do not disclose or suggest the up-regulation of class I-restricted antigen presentation which in turn generates an antigen-specific $CD8^+$ cytotoxic T cell response as disclosed by the present invention.

Applicants do not agree with the Examiner that the teachings of Ramoner *et al.* provide the skilled artisan with the either the motivation to combine the teaching of Ramoner *et al.* with the teaching of the '963 patent and/or Thurnher *et al.* to result in the present invention, nor does Ramoner *et al.* if it were combined with the '963 patent and/or Thurnher *et al.* disclose or suggest the present invention. Ramoner *et al.* merely review the data provided in Thurnher *et al.* and add the teachings that BCG induces the release of IL-8 and stimulates the allostimulatory potential of human dendritic cells. The release of IL-8 has not been associated with the induction of an antigen specific cytotoxic T cell response. In addition, it is well known to the skilled artisan that the MLR assay measures non-specific $CD4^+$ T cell activation as indicated by T cell proliferation and does not measure an antigen specific cytotoxic T cell response. As such, the teachings of Ramoner *et al.* either alone or in any combination with the '963 patent and/or Thurnher *et al.* merely suggest that dendritic cells matured in the presence of BCG and a tumor

associated antigen could be used in combination with BCG therapy. Further, if the teachings of the cited references are combined with Pithie *et al.* as discussed above the skilled artisan would have been lead to the result that at best the BCG matured dendritic cells would have induced a $CD4^+$ cytolytic response, whereas the present invention discloses and claims the up-regulation of a $CD8^+$ cytotoxic T cell response as a consequence of the up-regulation of class I-restricted antigen presentation. Applicants respectfully submit that a $CD8^+$ cytotoxic T cell response is typically considered in the art to be the most effective cell mediated immune response against tumor cells.

The Examiner has asserted that the disclosure at page 32, line 18, of the specification reciting, "Inciting a potent anti-tumor response using immunotherapy has been limited in efficacy partly due to difficulty in stimulating a cytotoxic T cell response" is precisely the reason for combining the methods of the prior art and optimizing the time of the combined method. Applicants believe that the remarks above demonstrate that at the time of the present invention the skilled artisan would not have been motivated to combine the methods of the prior art to disclose or suggest the present invention. The prior art as set forth in the specification had not been successful in inciting a potent anti-tumor response using immunotherapy because of the difficulty in stimulating an antigen specific cytotoxic T cell response. The teaching of Thurnher *et al.* and Ramoner *et al.* add nothing to methods for using dendritic cells to induce an antigen specific cytotoxic T cell response. The dendritic cells matured with BCG merely were shown to have up-regulated CD83 and CD86 expression, up-regulated IL-8 release and the ability to stimulate the allostimulatory potential of human dendritic cells, and down regulated antigen uptake. As set forth above, none of these characteristics has been associated with dendritic cells having increased MHC-class I expression or having the ability to induce an antigen specific cytotoxic T cell response. As disclosed by Pithie *et al.* above, administration of BCG induces a $CD4^+$ lysogenic response to macrophage infected with mycobacteria and an increase in CD86 expression has been associated with an increase in $CD4^+$ T cell activation. Therefore, the teachings of Ramoner *et al.* and Thurnher *et al.* either alone or in any combination with the '963 patent do not disclose or suggest the claimed invention. This conclusion is supported by the

literature as represented by Pithie *et al.* which actually predicts a lack of a $CD8^+$ cytotoxic T cell response in the context of BCG stimulation.

In view of the above amendments and remarks, Applicants respectfully request the Examiner to reconsider and withdraw the rejection of claims 10, 12-14, 16-21, and 36-43 under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent No. 5,788,963 ("the '963 patent") in view of Thurnher *et al.* and Ramoner *et al.*

Rejections Under 35 U.S.C. §112

Claims 40-43 remain rejected under 35 U.S.C. §112, first paragraph, the Examiner believing that the specification does not contain a written description of the claimed invention, in that the disclosure does not reasonably convey to one skilled in the relevant art that the inventor(s) had possession of the claimed invention at the time the application was filed. In particular, the Examiner does not believe that the specification and the claims as originally filed do not provide support for the invention as now claimed, specifically, the generic method of Claim 40 comprising contacting antigen with DCs subsequent to contact of DCs with BCG.

Applicants do not agree with the rejection of the Examiner, but in order to further expedite prosecution of certain subject matter disclosed and claimed in the instant application, claims 40 through 43 have been canceled without prejudice to further prosecution of the subject matter of the claims in a related copending application. Therefore, this rejection is moot.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance and an action to that end is respectfully requested. If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 206-467-9600.

Respectfully submitted,

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In Vivo CD86 Blockade Inhibits CD4$^+$ T Cell Activation, Whereas CD80 Blockade Potentiates CD8$^+$ T Cell Activation and CTL Effector Function[1]

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To address whether a functional dichotomy exists between CD80 and CD86 in naive T cell activation in vivo, we administered anti-CD80 or CD86 blocking mAb alone or in combination to mice with parent-into-F_1 graft-vs-host disease (GVHD). In this model, the injection of naive parental T cells into unirradiated F_1 mice results in either a Th1 cytokine-driven, cell-mediated immune response (acute GVHD) or a Th2 cytokine-driven, Ab-mediated response (chronic GVHD) in the same F_1 recipient. Combined CD80/CD86 blockade beginning at the time of donor cell transfer mimicked previous results seen with CTLA4Ig and completely abrogated either acute or chronic GVHD by preventing the activation and maturation of donor CD4$^+$ T cells as measured by a block in acquisition of memory marker phenotype and cytokine production. Similar results were seen with selective CD86 blockade; however, the degree of CD4 inhibition was always less than that seen with combined CD80/CD86 blockade. A more striking effect was seen with selective CD80 blockade in that chronic GVHD was converted to acute GVHD. This effect was associated with the induction of Th1 cytokine production, donor CD8$^+$ T cell activation, and development of antihost CTL. The similarity of this effect to that reported for selective CTLA4 blockade suggests that CD80 is a critical ligand for CTLA4 in mediating the down-regulation of Th1 responses and CD8$^+$ T cell activation. In contrast, CD86 is critical for the activation of naive CD4$^+$ T cells in either a Th1 or a Th2 cytokine-mediated response. *The Journal of Immunology,* **2002, 168: 3786–3792.**

Activation of naive T cells requires cognate interaction of the TCR with Ag as well as a second costimulatory signal. The best-characterized costimulatory molecules are CD28 and CTLA4 on the T cell and their coligands CD80 (B7-1) and CD86 (B7-2), expressed primarily on APCs. The importance of this costimulatory pathway in T cell activation is supported by in vitro data demonstrating that combined CD80 and CD86 blockade at the time of TCR engagement blocks T cell production of IL-2, IFN-γ, and IL-4 (1–4). Similarly, in vivo studies have shown that combined CD80 and CD86 blockade abrogates naive T cell activation and has proven to be beneficial in a variety of animal disease models in which T cell activation is critical, e.g., lupus, diabetes, experimental autoimmune encephalomyelitis, and allograft rejection (5–11). As a result, CD80 and CD86 have emerged as potential therapeutic targets in diseases mediated by T cells.

Although controversial, it has been suggested that CD80 and CD86 provide distinct signals for the differentiation of T cells (Th0) into either a Th1 (IFN-γ-, IL-2-producing) or a Th2 (IL-4-, IL-10-producing) cytokine phenotype (12). Results using selective CD80 or CD86 blockade in vivo have been contradictory. For example, Kuchroo et al. (13), using experimental autoimmune encephalomyelitis as a model of Th1-mediated disease, demonstrated that selective CD80 blockade ameliorated disease by shifting the cytokine phenotype to a Th2 pattern, whereas selective CD86 blockade worsened disease. It was concluded that in vivo Th1 responses require CD80 costimulation, whereas Th2 responses require CD86 costimulation. By contrast, Lenschow et al. (6), using the nonobese diabetic mouse, a model in which a Th1 phenotype predominates, observed that selective CD80 blockade worsened disease, whereas selective CD86 blockade had a beneficial effect, leading to the conclusion that CD86 was a major costimulatory ligand in a Th1 response. Although disease in both models is Th1 driven, the models differ in several important areas, among which are the use of adjuvants and the degree to which disease is mediated by naive and memory T cells. It is becoming increasing clear that differences exist in the dependence of memory and naive T cells on CD28 costimulation (14), and that recently identified costimulatory receptor-ligand pairs are important in the differentiation of previously activated T cells (15). However, CD80 and CD86 remain the major costimulatory molecules in the induction of a naive T cell response.

To address whether a functional dichotomy exists between CD80 and CD86 in naive T cell activation in vivo, we used the parent-into-F_1 (P→F_1)[3] model of graft-vs-host disease (GVHD). In this model, the injection of homozygous (parental) naive T cells into unirradiated F_1 mice results in either a Th1 cytokine-driven, cell-mediated immune response (acute GVHD) or a Th2 cytokine-driven, Ab-mediated response (chronic GVHD). Important features of the model are as follows: 1) the alloantigen-specific donor

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[3] Abbreviations used in this paper: P→F_1, parent-into-F_1; GVHD, graft-vs-host disease.

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T cells that drive disease can be studied separately from nonspecifically activated (host) T cells; 2) either a Th1-mediated or Th2-mediated response can be induced in the same F_1 recipient depending on the strain used for donor cells; and 3) in vivo manipulations that alter disease by blocking T cell activation can be readily distinguished from those that induce immune deviation. Our results indicate that CD86 is critical for naive $CD4^+$ T cell activation and differentiation into either a Th1 or Th2 phenotype. In contrast, CD80 is important in mediating a down-regulatory effect on $CD8^+$ CTL development, perhaps through preferential binding to CTLA4.

Materials and Methods

Mice

Six- to 8-wk-old C57BL/6 (B6), DBA/2 (DBA or D2), and $B6D2F_1$ mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Induction of GVHD

Single cell suspensions of splenocytes were obtained from either B6 or DBA male mice and resuspended in RPMI 1640 medium without FCS at 10^8 viable cells per milliliter. Unless otherwise noted, acute and chronic GVHD were induced by tail vein injection of either 50×10^6 B6 or 80×10^6 DBA splenocytes into nonirradiated $B6D2F_1$ mice, as previously described (16). Negative controls consisted of age- and sex-matched uninjected F_1 mice.

In vivo reagents and treatment protocol

Anti-CD80 mAb (16-10A1) and anti-CD86 mAb (GL1) were obtained from BD PharMingen (San Diego, CA). The anti-CD80-specific fusion protein Y100F (17) was a gift of Dr. R. Peach (Bristol-Myers Squibb, Princeton, NJ). Reagents were used at a dose of 100 μg of anti-CD80 mAb (16-10A1), 200 μg of Y100F, and 100 μg of anti-CD86 (GL1) mAb. Control mice received 100 μg of rat IgG2a κ (isotype control for anti-CD86) and either 100 μg of hamster IgG (control for anti-CD80 mAb) or 200 μg of L6, a mouse-human fusion Ab specific for L6 tumor Ag (control for Y100F). Reagents were administered i.v. on the day of parental cell transfer and on days 3 and 7.

Flow cytometry studies

At the specified time points mice were sacrificed and spleens were harvested. Splenocytes were first incubated with anti-murine FcγR mAb 2.4G2 (18) for 15–20 min, then stained with saturating concentration of FITC-conjugated, PE-conjugated, or biotin-conjugated mAb. Fluorochrome-conjugated anti-CD4, anti-CD8, anti-B220, anti-H-$2K^b$, anti-H-$2K^d$, and anti-CD44 were purchased from BD PharMingen. Three-color flow cytometry was performed using a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA). Lymphocytes were gated based on forward and side scatter. Donor $CD4^+$ and $CD8^+$ T cells were identified as cells staining positive for the respective T cell marker and negatively for MHC class I of the nondonor parent. Analysis of CD44 brightness was performed on donor-gated $CD4^+$ and $CD8^+$ T cells. Anti-CD44 staining gave a clearly distinguishable bimodal pattern, allowing separation of donor T cells into bright ($CD44^{high}$) and dull ($CD44^{low}$) subpopulations.

Serologic assays

Serum was tested by ELISA for the presence of anti-ssDNA IgG Abs (16). Briefly, microtiter plates were coated with heat-denatured salmon sperm DNA, blocked with 2% BSA-PBS, and then incubated with serial 2-fold dilutions of mouse serum beginning at a dilution of 1/40. Wells were then washed and incubated with anti-mouse IgG conjugated with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL). OD was determined at 405 nm. MRL/*lpr* serum was assayed as a standard, and arbitrary units were calculated using a value of 1000 U/ml for pooled MRL/*lpr* serum.

Detection of CTL activity ex vivo

Effector CTL activity was tested using freshly harvested splenocytes without an in vitro sensitization period in a 4-h ^{51}Cr release assay as described (19). Targets were ^{51}Cr-labeled EL-4 (H-2^b) or P815 (H-2^d) cell lines. Using serial dilutions, effectors were tested in triplicate at four E:T ratios beginning at 100:1 (1.5×10^6 effectors and 0.015×10^6 targets per well). The percentage of lysis was calculated according to the following formula: [(cpm sample − cpm spontaneous)/(cpm maximum − cpm spontaneous)] × 100%. Results are shown as the mean percent of lysis ± SEM at a given E:T ratio for each treatment group.

Cytokine expression by RT-PCR

The coupled RT-PCR was used as previously described (20). Briefly, 1×10^5 splenocytes were homogenized in RNA-Stat-60 (Tel-Test, Friendswood, TX). RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Life Technologies, Grand Island, NY). IL-4- and IFN-γ-specific primers were used as previously described (20). To ensure that equal amounts of mRNA were amplified, RT-PCR was performed using primers for the housekeeping gene hypoxanthine phosphoribosyl transferase. For each PCR product the optimal number of PCR cycles was determined experimentally. PCR products were separated on agarose gel, transferred to nitrocellulose, and probed with cytokine-specific probes conjugated with HRP (Oligos Etc., Wilsonville, OR). Blots were developed by ECL (Amersham, Little Chalfont, U.K.). Bands were imaged by autoradiography and quantitated by densitometry. Cytokine densitometry results for each sample were normalized to hypoxanthine phosphoribosyl transferase and results for each cytokine were calculated as fold increase over the respective cytokine expression in control F_1 mice according to the ratio (normalized experimental group mean:normalized untreated F_1 mean).

Statistical analysis

Data were examined for normality and equal variance (Kolmogorov-Smirnov). If satisfactory, groups were compared by a two-tailed Student's *t* test; if not, they were compared by the Mann-Whitney rank sum test.

Results

Maximal inhibition of chronic GVHD-associated B cell expansion is achieved with selective CD80 blockade

To determine the respective roles of CD80 and CD86 in the development of an in vivo Th2-driven response (e.g., chronic GVHD), combined or selective CD80 and CD86 blockade was initiated at the time of parental cell transfer, followed by analysis at day 14. As shown in Table I, untreated or control Ig-treated chronic GVHD mice exhibit the expected ≥2-fold increase in host B cell numbers compared with normal F_1 mice, similar to previous reports (21). Significant reductions in B cell expansion were seen for all GVHD groups with CD80 and/or CD86 blockade ($p < 0.01$), with the strongest effect seen with CD80 blockade. Selective CD86 blockade reduced GVHD-associated B cell expansion by 40–60% compared with untreated or control Ig-treated chronic GVHD. Further inhibition was seen with combined CD80/CD86 blockade, nearly normalizing host B cell numbers ($p = 0.03$, normal F_1 vs CD80/86 blockade). The most striking results were seen for selective CD80 blockade, in which B cell numbers were reduced to ~50% below those of normal F_1 mice ($p < .001$), suggesting that CD80 blockade may not just prevent B cell expansion but may actually promote B cell elimination. This observation has been confirmed in three additional independent experiments (B cell numbers range 15–50% below normal F_1 controls) and was seen regardless of whether CD80 blockade was achieved using Y100F or anti-CD80 mAb (data not shown). Although the reduction in B cell expansion following combined CD80/CD86 blockade was typically greater than that seen with CD86 blockade alone, B cell numbers were never reduced below those of normal F_1 mice as seen with selective CD80 blockade. Normal F_1 mice treated with anti-CD80 mAb alone using the same dosing regimen exhibited no reduction of B cells at day 14 compared with untreated F_1 mice (data not shown).

Only combined CD80/CD86 blockade completely inhibits autoantibody production

Serum anti-ssDNA levels were used as a marker for the polyclonal B cell hyperactivity characteristically present in chronic GVHD mice and transiently in acute GVHD mice (16). Selective CD80 or

Table I. *CD80 blockade in chronic GVHD promotes donor CD8$^+$ T cell expansion and B cell elimination, whereas CD86 blockade inhibits donor CD4$^+$ T cell and host B cell expansion*[a]

Group	Donor T Cell		B Cells	Anti-ssDNA[b]
	CD4$^+$	CD8$^+$		
Normal F_1	NA[c]	NA	50.6 ± 2.9	15 ± 3
cGVHD + no treatment	2.7 ± 0.5	0.5 ± 0.2	102.3 ± 6.2	69 ± 13
cGVHD + anti-CD80 mAb	3.2 ± 0.5	4.1 ± 0.6[d]	23.2 ± 4.0[d]	42 ± 13[d]
cGVHD + anti-CD86 mAb	1.8 ± 0.1[d]	0.5 ± 0.1	79.3 ± 2.5[d]	44 ± 17[d]
cGVHD + anti-CD80/CD86	1.2 ± 0.3[e]	0.9 ± 0.2	68.0 ± 5.8[d]	11 ± 1[e]
cGVHD + Control Ig	3.7 ± 0.2	0.8 ± 0.1	118.1 ± 6.6	75 ± 9

[a] Chronic GVHD (cGVHD) was induced as described in *Materials and Methods* and mice either were untreated or received anti-CD80 mAb, anti-CD86 mAb, both anti-CD80 and anti-CD86, or control Ab (L6 plus rat isotype control) on days 0, 3, and 7 after parental cell transfer. Splenocytes were analyzed by flow cytometry at 14 days after parental cell transfer. Values for lymphocyte subsets are shown as group mean ± SE × 10^{-6}. n = 5 mice per group.

[b] Serum units per milliliter ± SE at day 14.

[c] NA, Not applicable.

[d] Values of $p < 0.01$ vs untreated chronic GVHD.

[e] Values of $p < 0.05$ vs anti-CD80 or anti-CD86 alone.

CD86 blockade resulted in a significant, but incomplete, reduction of serum anti-ssDNA titers compared with control Ig-treated mice ($p < 0.01$) (Table I). Complete normalization of anti-ssDNA levels was only seen with combined CD80/CD86 blockade ($p < 0.05$, combined CD80/86 blockade vs CD86 or CD80 blockade alone). Taken together, our results with combined CD80/CD86 blockade are similar to those reported for CTLA4Ig (22) and indicate that, at the doses used, complete costimulatory blockade is achieved with the combination of blocking Abs but not with either of these agents alone.

CD86 blockade inhibits but CD80 blockade promotes donor T cell engraftment

As shown in Table I, selective CD86 blockade reduced engraftment of donor CD4$^+$ T cells by ~40% compared with control Ig-treated chronic GVHD; however, combined CD80/CD86 blockade resulted in a nearly 50% further reduction in donor CD4$^+$ T cell engraftment ($p < 0.05$, average additional reduction of 50% over four experiments for CD86 vs CD80/86), implying that both CD80 and CD86 contribute to donor T cell engraftment and expansion. In contrast, no reduction of donor CD4$^+$ T cell engraftment was seen following selective CD80 blockade, but rather an increase in engraftment was observed (range of 20–80% increase in CD4$^+$ T cell engraftment with CD80 blockade compared with untreated chronic GVHD; n = 3 experiments). Strikingly, donor CD8$^+$ T cell engraftment was increased 8-fold compared with control Ig-treated or untreated chronic GVHD mice (Table I) and was seen using either anti-CD80 mAb or Y100F in three additional experiments (range, 3- to 10-fold increase; data not shown).

CD80 blockade converts chronic GVHD to acute GVHD

The enhanced donor CD8$^+$ T cell engraftment and reduced host B cell numbers following selective CD80 blockade suggest that CD8$^+$ donor antihost CTL are present and that acute GVHD has developed in these mice. Key features that differentiate acute from chronic GVHD are the presence of ex vivo antihost CTL activity and elevated IFN-γ production, both of which are present in acute GVHD and absent in chronic GVHD (21). As shown in Fig. 1, chronic GVHD mice receiving selective CD80 blockade exhibit significant antihost CTL activity ex vivo, which is not observed for either untreated or control mAb-treated chronic GVHD mice (CD80 blockade vs control Ig-treated, $p < 0.01$; normal F_1 vs chronic GVHD or chronic GVHD plus L6, p = NS). Results in two additional experiments yielded a range in the percentage of specific killing of 10–27.4% with CD80 blockade vs 2–9.2% for control mice (n = 15 mice per treatment). Also consistent with acute GVHD, CD80 blockade in chronic GVHD mice resulted in a significant increase (~4-fold) in IFN-γ mRNA compared with all other groups ($p < 0.05$) (Fig. 2*B*). By contrast, the increased IL-4 expression typical of chronic GVHD (21) was absent in anti-

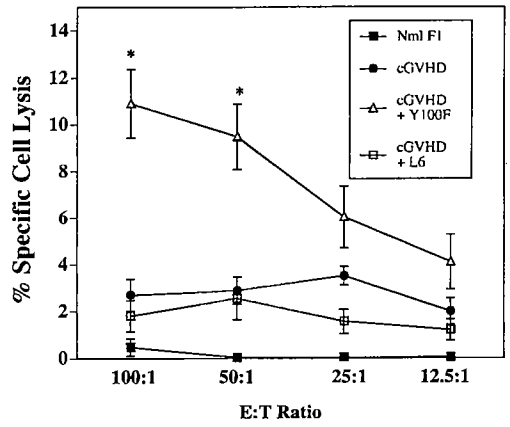

FIGURE 1. Selective CD80 blockade in chronic GVHD mice induces antihost cytolytic activity. Groups consisted of untreated normal F_1 mice or chronic GVHD mice receiving either no mAb, 200 μg of Y100F, or 200 μg of control mAb (L6) on days 0, 3, and 7 after parental cell transfer (n = 4–5 mice per group). Ex vivo antihost CTL activity was determined on day 10. Results are shown as group mean ± SE percentage of killing on H-2^b targets at a given E:T ratio; *, $p < 0.01$.

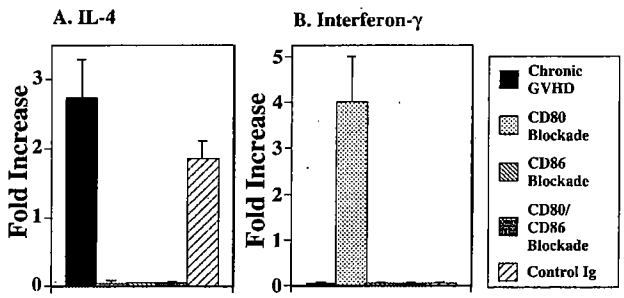

FIGURE 2. Selective CD80 blockade promotes IFN-γ mRNA expression in chronic GVHD mice. Semiquantitative RT-PCR was performed on cDNA from splenocytes taken on day 14 from chronic GVHD mice treated as outlined in Table I. Results are shown as average fold increase over uninjected F_1 mice for IL-4 (*A*) and IFN-γ (*B*) (n = 5 mice per group).

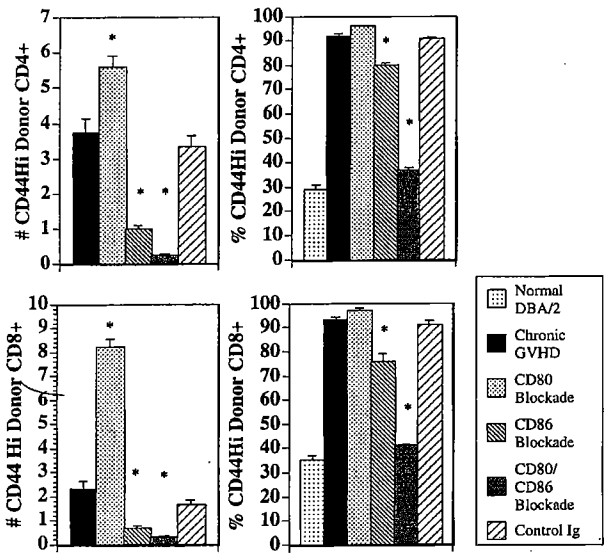

FIGURE 3. Both selective CD86 blockade and combined CD80/CD86 blockade inhibit donor $CD4^+$ T cell activation, whereas selective CD80 blockade promotes activation of donor $CD8^+$ T cells in chronic GVHD. Mice with chronic GVHD received no treatment or either 200 μg Y100F, 100 μg of anti-CD86, combined Y100F and anti-CD86, or L6 plus rat isotype control at days 0, 3, and 7. On day 14, donor $CD4^+$ (*upper panels*) and $CD8^+$ (*lower panels*) T cells were assayed for CD44 up-regulation by flow cytometry. $CD44^{high}$ values are shown as mean percentage (*right panels*) or mean absolute number (*left panels*) per group ± SE ($n = 5$ mice per group). *, $p < 0.01$.

CD80-treated mice (Fig. 2*A*). CD86 blockade and combined CD80/86 blockade were equally effective in blocking IL-4 production in chronic GVHD mice and did not result in the induction of IFN-γ.

Donor T cell activation in chronic GVHD is inhibited by CD86 blockade but enhanced by CD80 blockade

The foregoing data strongly suggest that CD86 blockade either alone or combined with CD80 blockade inhibits donor T cell activation, whereas selective CD80 blockade does not inhibit T cell activation but rather induces immune deviation. To address the activation status of donor T cells following costimulatory blockade, donor T cell expression of an activation marker, CD44, was measured (23, 24). It has been previously shown that, in this model, >90% of donor T cells exhibit memory cell phenotype ($CD44^{high}$ and $CD69^{low}$) by 14 days after cell transfer, and that complete costimulatory blockade with CTLA4Ig treatment prevents donor T cell activation, expansion, and acquisition of memory phenotype (22). As shown in Fig. 3, combined CD80/CD86 blockade in chronic GVHD mice acts similarly to published results with CTLA4Ig in that it completely inhibits the increase in both the percentage and the number of $CD44^{high}$ donor $CD4^+$ T cells ($p < 0.01$). Smaller but statistically significant reductions in the percentage and number of donor $CD44^{high}CD4^+$ T cells were also observed with selective CD86 blockade ($p < 0.01$, CD86 blockade vs control Ig). In contrast, selective CD80 blockade significantly increased not only the number of $CD44^{high}$ donor $CD4^+$ T cells ($p < 0.01$) but also the number of $CD44^{high}$ donor $CD8^+$ T cells compared with untreated chronic GVHD mice, consistent with enhanced donor $CD4^+CD8^+$ T cell expansion and CTL maturation induced by selective CD80 blockade. The few $CD8^+$ T cells that engraft in untreated chronic GVHD display an activated phenotype that is blocked by CD86 blockade and combined CD80/CD86 blockade.

CD80 and/or CD86 blockade in acute GVHD mimics the effects seen in chronic GVHD

To determine whether the strikingly different effects of selective CD80 and CD86 blockade seen in a Th2/Ab-mediated response (chronic GVHD) are also seen in a Th1/cell-mediated response, selective costimulatory blockade was produced in acute GVHD mice. As shown in Table II, using 50×10^6 B6 donor splenocytes, combined CD80/CD86 blockade markedly impaired donor $CD4^+$ and $CD8^+$ T cell engraftment and completely blocked the elimination of host B cells, characteristic of acute GVHD. These results are similar to those reported for CTLA4Ig treatment in acute GVHD (22, 25) and indicate that, taken together with the above results, combined CD80/CD86 administration is capable of complete costimulatory blockade of donor T cells in either a Th1- or Th2-driven response. Selective CD86 blockade was only marginally effective in blocking acute GVHD, as shown by a small but statistically significant improvement in host B cell survival compared with control Ig-treated acute GVHD mice (8.6×10^6 vs 4.5×10^6; $p < 0.05$).

No inhibition of acute GVHD was seen with selective CD80 blockade, and instead low-level potentiation of disease was observed as measured by small but statistically significant further reduction in host B cells (compared with control Ig-treated or untreated acute GVHD ($p < 0.05$)). Because acute GVHD induced with 50×10^6 donor cells results in near maximal elimination of host B cells, potentiation is difficult to assess. A second experiment was then performed using fewer donor cells (40×10^6) to determine whether a modulatory effect of selective CD80 or CD86 blockade could be detected. As shown in Table II, despite the

Table II. *Combined CD80/CD86 blockade inhibits acute GVHD more effectively than selective CD86 blockade*[a]

	Donor					
	Expt. 1			Expt. 2		
Group	$CD4^+$	$CD8^+$	B cells	$CD4^+$	$CD8^+$	B cells
Normal F_1	ND[b]	ND	41.8 ± 2.6	ND	ND	44.9 ± 4.08
aGVHD	1.4 ± 0.2	2.3 ± 0.3	2.9 ± 0.3	1.1 ± 0.1	1.8 ± 0.2	1.7 ± 0.2
aGVHD + Y100F	0.9 ± 0.1	1.5 ± 0.1[c]	2.4 ± 0.4	0.5 ± 0.1	0.7 ± 0.1[c]	0.8 ± 0.1[c]
aGVHD + anti-CD86	0.6 ± 0.2	1.4 ± 0.4[c]	8.6 ± 1.3[c]	0.3 ± 0.03[c]	0.1 ± 0.01[c]	45.3 ± 2.2[c]
aGVHD + Y100F/anti-CD86	0.2 ± 0.03[c]	0.2 ± 0.03[c]	45.3 ± 4.1[c]	0.3 ± 0.05[c]	0.3 ± 0.06[c]	53.3 ± 4.4[c]
aGVHD + control Ig	0.9 ± 0.1	3.1 ± 0.4	4.5 ± 0.4	0.8 ± 0.2	1.2 ± 0.08	3.1 ± 0.2

[a] Acute GVHD (aGVHD) was induced by injecting either 50×10^6 (Expt. 1) or 40×10^6 (Expt. 2) B6 splenocytes. Dosing of mAb is as in Table I and *Materials and Methods*. Mice were sacrificed on day 14 and lymphocyte subsets were analyzed by flow cytometry. Results are expressed as the group mean ± SE $\times 10^{-6}$. $n = 5$ mice per group.

[b] ND, Not detectable above background.

[c] Values of $p < 0.05$ vs untreated acute GVHD.

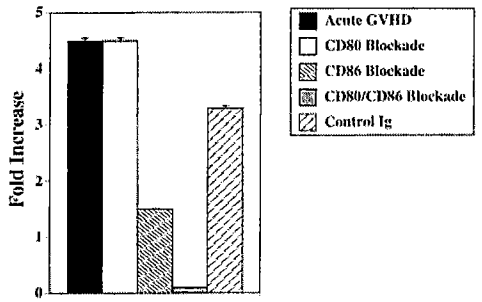

FIGURE 4. CD86 blockade alone or combined with CD80 blocks IFN-γ mRNA expression in acute GVHD mice. Acute GVHD was induced using 40 × 10^6 B6 splenocytes and F_1 mice were treated as in Table II. RT-PCR was performed as in *Materials and Methods* using day 14 splenocytes. Results are shown as average fold increase over normal F_1 mice (n = 5 mice per group).

reduced number of donor cells, near total elimination of host B cells was still observed for untreated or control Ig-treated acute GVHD mice. Nevertheless, acute GVHD was blocked not only by combined CD80/CD86 blockade but also by selective CD86 blockade, as evidenced by complete inhibition of host B cell elimination (Table II), inhibition of IFN-γ up-regulation (Fig. 4), and an inhibition of CD44 up-regulation on donor $CD4^+$ and $CD8^+$ T cells as measured by either percentage or absolute number (Fig. 5), although selective CD86 blockade was less effective in blocking CD44 up-regulation than combined CD80/CD86 blockade. A potentiating effect of selective CD80 blockade was again difficult to detect due to the profound elimination of host B cell, even at the reduced donor cell inoculum, and is better seen in the chronic GVHD model. It should be noted that detection of donor T cells by flow cytometry becomes difficult after 2 wk in untreated acute GVHD mice, due to an overall down-modulation of MHC expression (26) and/or acquisition of host MHC by donor cells (27) that parallels disease severity (28). We interpret the reduced numbers of donor $CD8^+$ T cells at 2 wk in anti-CD80 mAb-treated mice (compared with control Ig-treated or untreated acute GVHD) to be

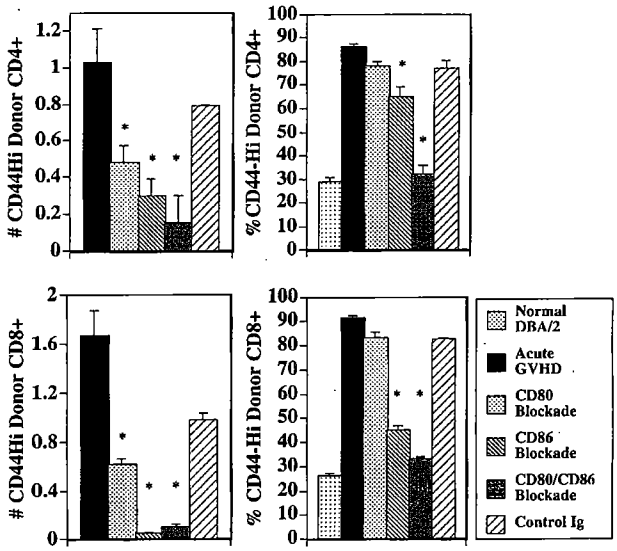

FIGURE 5. Both selective CD86 blockade and combined CD80/CD86 blockade inhibit donor $CD4^+$ T cell activation in acute GVHD. Acute GVHD was induced with 40 × 10^6 B6 splenocytes and mAb dosing is as described in Table II. CD44 up-regulation on donor $CD4^+$ and $CD8^+$ T cells was determined on day 14 by flow cytometry. Results are displayed as described for Fig. 3 (n = 5 mice per group). *, $p < 0.01$ vs acute GVHD.

a reflection of accelerated GVHD-associated MHC down-regulation rather than impaired engraftment and milder disease. In support of this, other parameters of acute GVHD such as host B cell elimination (Table II), IFN-γ gene expression (Fig. 4), and percentage of donor CD4/$CD8^{high}$ cells are either comparable to untreated acute GVHD or significantly worse (e.g., host B cells, $p <$ 0.05). Importantly, the observation that IFN-γ levels are not reduced in anti-CD80-treated acute GVHD compared with untreated acute GVHD mice supports our interpretation that donor T cells are not, in fact, reduced by anti-CD80 treatment but rather MHC down-regulation is accelerated and, by extension, acute GVHD is accelerated.

Discussion

Recently, new members of the B7 costimulatory family and their receptors have been described in addition to CD80 and CD86 (15, 29, 30). However, our results underscore the critical importance of CD80 and CD86 in the activation of naive $CD4^+$ T cells. In this report, we have used the P→F_1 model of GVHD to determine the in vivo role of CD80 and CD86 in the initiation of either a Th1/cell-mediated response (acute GVHD) or a Th2/Ab-mediated response (chronic GVHD). Advantages of this model are as follows: 1) either form of GVHD can be induced in the same F_1 depending on the T cell subsets injected (16, 31, 32); 2) the Ag-specific T cells driving disease can be monitored separately from the remainder of the T cell pool; and 3) in vivo manipulations that alter disease by blocking T cell activation can be readily distinguished from those that cause alterations in the phenotype of the immune response (immune deviation). Using this model, we observed that combined CD80/CD86 blockade completely inhibited donor $CD4^+$ T cell expansion, cytokine production, and acquisition of an activation phenotype in both acute and chronic GVHD. CD28/B7 costimulation has been reported to be critical for naive T cell differentiation for Th2 cells but not necessarily for Th1 cell development (33, 34); however, in the data presented in this work, combined CD80/CD86 blockade completely blocked both Th1-driven acute GVHD and Th2-driven chronic GVHD. Moreover, our results are in agreement with previous studies demonstrating that naive T cell activation can be prevented by blockade of both CD80 and CD86 using either CTLA4Ig, a fusion protein that binds CD80 and CD86 with high affinity (35), or combined anti-CD80 and anti-CD86 mAb. For example, combined anti-CD80/CD86 mAb treatment has been shown to inhibit acute lethal GVHD in an irradiated recipient model following transfer of either purified $CD4^+$ or $CD8^+$ T cells (36) and to block the Ag-specific expansion and activation of adoptively transferred TCR-transgenic $CD4^+$ T cells (37). Similarly, CTLA4Ig was reported to completely inhibit both acute and chronic GVHD in the P→F_1 model (22, 25). The mechanism involved appeared to be the induction of anergy rather than clonal deletion, because donor T cells were not deleted following CTLA4Ig treatment; however, they did not produce IL-2, nor did they acquire an activation phenotype ($CD44^{high}$) (22).

Our studies with selective costimulatory blockade underscore the dichotomous roles of CD28 and CTLA4, as well as their preferential interactions with CD80 or CD86. CD28 is constitutively expressed on T cells and upon engagement with CD80 and CD86 delivers a proliferative signal to Ag-specific T cells, whereas CTLA4 is not expressed on resting T cells but instead is induced upon activation and delivers a down-regulatory signal to proliferating T cells (38, 39). The critical role of CD28 in donor T cell activation has been reported by Yu et al. (40), whose study demonstrated that anti-CD28 mAb treatment prevented acute GVHD in

an irradiated recipient model. Our results demonstrating that selective CD86 blockade is almost as effective as combined CD80/86 blockade in blocking donor T cell activation in either form of GVHD, whereas selective CD80 blockade has no detectable inhibitory effect, indicate that in the P→F_1 model CD86 is the primary ligand for CD28 in the initial activation of naive donor $CD4^+$ T cells.

By contrast, our results demonstrating that selective CD80 blockade converts chronic GVHD to acute GVHD support the hypothesis that CD80 is a major ligand for CTLA4 in the delivery of a down-regulatory signal for Th1 responses. Regarding the paradoxical results seen with selective CD80 blockade (conversion of chronic GVHD to acute GVHD), it is important to note that in the DBA→F_1 model of chronic GVHD, donor $CD8^+$ T cells are contained in the donor inoculum; however, they typically do not engraft or become activated in numbers sufficient to induce acute GVHD. As a result, donor $CD4^+$ T cell-driven chronic GVHD ensues. The defect in DBA $CD8^+$ antihost CTL generation has not been fully explained but is due in part to a 9- to 10-fold reduction in the anti-F_1 pCTL frequency compared with that of B6 mice (16). Thus, the DBA→F_1 model of chronic GVHD allows the identification of biologic agents that will potentiate $CD8^+$ CTL development in vivo and as a result convert chronic GVHD to acute GVHD. We have previously observed that rIL-12 administration, like selective CD80 blockade, converts chronic GVHD to acute GVHD in DBA→F_1 mice (41). However, with rIL-12 administration, engraftment and expansion of donor $CD8^+$ T cells, although increased over control, were not increased to the degree observed with CD80 blockade, suggesting that rIL-12 promotes CTL effector function in the engrafted donor DBA $CD8^+$ T cells but does not significantly promote their expansion. In contrast, selective CD80 blockade in our studies was shown to promote DBA $CD8^+$ T cell activation, expansion, and maturation of antihost CTL effectors, consistent with either the delivery of a stimulatory signal or the loss of an inhibitory signal.

It has been well reported that in vivo CTLA4 blockade potentiates Th1-mediated responses due to the loss of a down-regulatory signal (42–45). In particular, CTLA4 blockade in the BALB→CBF_1 model of chronic GVHD has been shown to enhance donor $CD8^+$ T cell expansion (46). Additionally, in a murine model of cardiac allograft rejection using CD28 knockout recipient mice, blockade of CTLA4 or CD80 blockade potentiated graft rejection, whereas CD86 blockade significantly prolonged allograft survival (47). It has been suggested that CTLA4 engagement preferentially limits Th1 differentiation (39); however, in other experimental systems CTLA4 blockade was reported to potentiate Th2 responses (48, 49), indicating that despite the demonstrated ability of CTLA4 to down-regulate T cell responses there is no inherent capacity of CTLA4 to differentially regulate Th1 vs Th2 development (39). Nevertheless, our results with selective CD80 blockade are remarkably similar to those seen with selective CTLA4 blockade, in that enhanced donor $CD8^+$ T cell expansion and activation are induced with treatment. In vitro and in vivo studies have identified CD80 as the preferential ligand for CTLA4 (47, 50, 51), and CD80-CTLA4 ligand binding in the absence of a functional CD28 molecule inhibits in vitro T cell activation (52). Taken together, these results strongly support the idea that CD80 is the major ligand for CTLA4 in vivo in the delivery of a T cell down-regulatory signal and that the mechanism by which CD80 blockade converts chronic GVHD to acute GVHD is through the interruption of CTLA4-CD80 interactions and the loss of that down-regulatory signal.

It is not clear whether selective CD80 blockade enhances donor $CD8^+$ T cell expansion: 1) directly through an effect on $CD8^+$ T cells; 2) indirectly through an effect on $CD4^+$ T cells, which in turn provide increased help to donor $CD8^+$ T cells; or 3) a combination of the two. This question will be difficult to directly address in the P→F_1 model, as donor $CD8^+$ T cells do not expand or mature into antihost CTL in the absence of donor $CD4^+$ T cell activation (32). Moreover, we have been unable to demonstrate significant donor $CD8^+$ T cell engraftment in F_1 mice receiving purified DBA $CD8^+$ T cells with or without selective CD80 blockade (data not shown), indicating that selective CD80 blockade cannot bypass the requirement for $CD4^+$ T cell help, most likely because, in the absence of donor $CD4^+$ T cell activation, significant up-regulation of CTLA4 on donor $CD8^+$ T cells does not occur. Our data demonstrating that selective CD80 blockade often results in a 125–200% increase in donor $CD4^+$ T cell engraftment (Table I and T. J. Lang, unpublished data) suggest a direct enhancing effect on donor $CD4^+$ cells. However, simply doubling the number of donor cells (to include $CD4^+$ T cells) in the DBA→F_1 model is usually not sufficient to convert chronic GVHD to acute GVHD (53). Thus, selective CD80 blockade probably promotes $CD8^+$ T cell expansion through both direct and indirect effects.

This study supports the primary role of CD86 in the differentiation of both Th1 and Th2 responses and is the first to show that selective CD80 blockade results in de novo expansion and maturation of $CD8^+$ CTL from inactive precursors, thereby converting chronic GVHD (Th2 mediated) to acute GVHD (Th1 mediated). These results suggest a potential novel therapeutic role for selective CD80 blockade. Currently, CTLA4 blockade is being evaluated for its therapeutic potential as an antitumor agent (39). A similar approach with selective CD80 blockade may be possible either alone or in combination with CTLA4 blockade.

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Generation of cytolytic T cells in individuals infected by *Mycobacterium tuberculosis* and vaccinated with BCG

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Abstract

Background **Macrophage activation by cytokines provides only a partial explanation of antimycobacterial immunity in man. Because cytolytic T lymphocytes have been shown to contribute to immunity in animal models of intracellular infection, the generation of mycobacterial antigen specific cytotoxic T cells was examined in the peripheral blood of patients with tuberculosis.**

Methods **Subjects comprised 36 patients with active tuberculosis (18 newly diagnosed) and 32 healthy volunteers, of whom 25 had had BCG vaccination and seven were Mantoux negative. The ability of purified protein derivative (PPD) stimulated peripheral blood lymphocytes to lyse autologous, mycobacterial antigen bearing macrophages was examined by using a chromium 51 release assay.**

Results **PPD stimulated lymphocytes from normal, Mantoux positive, BCG vaccinated subjects produced high levels of PPD specific cytolysis, whereas lymphocytes from unvaccinated, uninfected subjects caused little or no cytolysis. The generation of cytolytic T lymphocytes by patients with tuberculosis was related to their clinical state. Those with cavitating pulmonary disease or lymph node tuberculosis generated PPD specific lymphocytes with cytotoxic ability similar to that of those from Mantoux positive control subjects, whereas lymphocytes from patients with non-cavitating pulmonary infiltrates showed poor antigen specific cytolysis. After seven days of stimulation with PPD in vitro, lymphoblasts contained both $CD4^+$ and $CD8^+$ cells. Mycobacterial antigen specific cytolysis was restricted to the $CD4^-$ cell population and was blocked by monoclonal antibodies directed against major histocompatibility class II (MHC) antigens.**

Conclusion **$CD4^+$ cytolytic T cells can lyse autologous macrophages presenting mycobacterial antigen and were found in patients with cavitating pulmonary tuberculosis or tuberculous lymphadenitis and in normal, Mantoux positive control subjects. The ability to generate these T cell responses seems to be a marker for response to mycobacteria and may contribute to tissue damage in tuberculosis. These responses do not provide protective immunity against *Mycobacterium tuberculosis* but may help in disease localisation.**

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Tuberculosis remains a major global health problem, especially, though not exclusively, in developing countries. The global estimate of the number of people with tuberculosis is approximately 50–60 million,[1] with eight to 10 million new cases each year.[2] Although mortality figures have been falling steadily over the past three decades, tuberculosis is recognised as a complication of T cell deficiency states, in particular of HIV infection.[3] Patients with AIDS are a hundred times more likely to contract tuberculosis than people without this disease.[4] This situation is of obvious importance in countries where tuberculosis and AIDS are already prevalent.

The causative agent, *Mycobacterium tuberculosis*, is a facultative intracellular pathogen that can replicate within human monocytes and macrophages. Protective immunity to tuberculosis depends on the interaction of T lymphocytes and mononuclear phagocytes,[5] while hypersensitivity to mycobacterial antigens (also mediated by T lymphocytes) contributes to the typical caseous tissue destruction. Protective immunity and hypersensitivity are clearly related but the detailed cellular mechanisms have not been fully explained. As a result of the work of Mackaness[6] it has been generally accepted that activation of macrophage antimycobacterial mechanisms by T cell secreted lymphokines such as gamma interferon is the cornerstone of protective immunity. Mycobacterial growth in mice is readily inhibited by lymphokine activated macrophages.[7,8] Activation of human macrophages with a variety of recombinant lymphokines, however, achieves modest inhibition of mycobacterial growth at best and is unlikely to fully explain antimycobacterial immunity.[9–12]

The generation of cytolytic T cells in response to intracellular bacterial pathogens, including mycobacteria, has been shown in animals.[13] Mycobacterial antigen specific cytolytic T cells are able to lyse autologous antigen bearing macrophages in murine tuberculosis. Cytolytic T cells have also been shown to inhibit the intracellular growth of *M tuberculosis* in a manner independent of interferon.[14]

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Furthermore, selective depletion of $CD8^+$ cytotoxic T cells increases the susceptibility of mice to infection with virulent *M tuberculosis*.[15] In man, mycobacterial antigen specific cytolytic T cells have been generated from the peripheral blood of BCG vaccinated subjects,[16 17] from patients with leprosy,[18 19] and from those with tuberculosis.[20] There has been speculation, therefore, about the function of cytolytic T cells in the immune response to tuberculosis. We have investigated the cytolytic activity of purified protein derivative (PPD) stimulated lymphocytes from patients and normal control subjects.

Methods

PATIENTS AND CONTROL SUBJECTS

Thirty six patients with bacteriologically or histopathologically proved tuberculosis were recruited from East Birmingham and Dudley Road Hospitals, Birmingham. The clinical details were documented and radiographs reviewed by one individual (ADP). Eighteen patients had newly diagnosed tuberculosis and had received less than two weeks' treatment when tested; the remaining 18 patients had received treatment for more than two weeks. Seventeen patients had localised cavitating pulmonary tuberculosis, eight had extrathoracic lymph node tuberculosis, six had pulmonary disease with subapical diffuse pulmonary involvement and little or no evidence of localised cavitation (diffuse, non-cavitating tuberculosis), and three had miliary tuberculosis. One patient had abdominal tuberculosis and one had pleural disease. One patient who presented initially with diffuse pneumonic disease but later developed diffuse lymphadenopathy was tested on both occasions. Five of the patients had lymphadenopathy and five developed an abscess that required needle aspiration or surgical drainage. No patient had known risk factors for HIV infection or any other disease associated with immunosuppression. Three patients had diabetes (one insulin dependent, two non-insulin dependent).

Thirty two healthy volunteers acted as control subjects. Twenty five had previously received BCG vaccination and were known to be Mantoux positive; they were not retested for this study. The size of the tuberculin reaction was not known in most of the subjects. Seven subjects had no history of BCG vaccination or exposure to tuberculosis and were Mantoux negative.

TISSUE CULTURE MEDIA

Complete medium (RPMI) consisted of RPMI 1640 (Gibco Biocult, Paisley) supplemented with glutamine (2 mM), penicillin (100 U/ml), and gentamicin (50 U/ml).

SEPARATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS

Peripheral blood cells were separated from defibrinated blood by Ficoll-Hypaque centrifugation, washed three times in RPMI and resuspended at 10^6 mononuclear cells per ml in RPMI with 10% autologous serum.

GENERATION OF STIMULATED T BLASTS (EFFECTOR CELLS)

Isolated peripheral blood cells were incubated in 2 ml volumes (1×10^6 cells/well) in 24 well tissue culture plates (Cell Cult) at 37°C, in 5% CO_2 in air for seven days. PPD (Statens Seruminstitute, Denmark) at 10 μg/ml final concentration was added at the beginning of the culture period to generate antigen specific effector cells. Interleukin 2 stimulated cells were prepared as above except that 20 U/ml recombinant Interleukin 2 were added instead of PPD at the beginning of culture Interleukin 2 was used as a non-specific T cell stimulant.

SURFACE MARKER ANALYSIS

The surface phenotype of lymphoblast cultures stimulated by antigen for seven days was determined by a sensitive rosette assay using monoclonal antibody coated indicator red cells[35] or by flow cytometry by Becton-Dickinson FACScan. Data were collected using the consort 30 program and analysed with the LYSYS program. The following monoclonal antibodies were used, CD3 (OKT3 obtained from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852), CD4 (BMA 040, Behringwerke AG, Marburg, Germany), CD8 (B941 a kind gift of Dr C Mawas, Marseilles, France), TCR-delta (T-cell Sciences, Boston, USA).

PREPARATION OF MACROPHAGE TARGETS

Freshly isolated cells were distributed in 150 μl volumes ($1{\cdot}5 \times 10^5$ cells/well)in 96 well round bottom microtitre tissue culture plates (Flow). We and others have previously shown that approximately 10% of the added mononuclear cells adhered as monocytes and this number was used to compute effector:target ratios in cytotoxicity assays.[20 21] After six days' incubation, the non-adherent cells were washed off and the adherent cells incubated with antigen (PPD 25 μg/ml or streptokinase/streptodornase (SK/SD) 250 U/ml as control antigen and labelled with chromium-51 (2 μCi/well, CJS-1, Amersham International) overnight in a final volume of 100 μl. After 16–24 hours incubation the plates were washed three times with warm RPMI containing 5% heat inactivated, group A positive human serum after which the wells were replenished with 50 μl of RPMI containing 10% human serum.

CYTOTOXICITY ASSAY

This method has been described in detail elsewhere.[18 20] Briefly, effector cells stimulated with antigen for seven days were harvested from the 24 well plates, washed once, and resuspended in RPMI supplemented with 10% human serum at 3×10^6/ml and $7{\cdot}5 \times 10^5$/ml. One hundred μl of these suspensions were added to target macrophages in triplicate to give effector:target ratios of approximately 20:1 and 5:1. Triplicate wells with 100 μl of medium were used to determine spontaneous isotope release.

Some patients with tuberculosis were so profoundly lymphopenic that very low numbers of effector and target cells were available.

For these patients only one effector:target cell ratio was tested for each antigen combination.

After 18 hours' incubation the supernatant was aspirated from individual wells into counting tubes. One hundred μl of 1% Triton X-100 (Sigma) were added to each cell pellet and incubated for 45 minutes at 60°C to lyse the remaining adherent cells. The entire volume was aspirated subsequently and transferred to further counting tubes. Activity of both the supernatant and pellet was assessed by measurement of radioactive decay with a gamma counter (LKB).

The percentage isotope release for each well was calculated by using the formula:

$$\% \text{ Isotope release} = \frac{\text{cpm supernatant}}{\text{cpm supernatant} + \text{cpm pellet}}.$$

The level of cytotoxicity in each well was determined as follows:

Cytotoxicity =
% isotope release in test well −
% isotope release in spontaneous release well.

Results are presented as the mean (SD) of triplicate estimates of the percentage ^{51}Cr release. The SD between triplicates was not normally greater than 5%. Spontaneous release in these assays was usually no greater than 20% of the total uptake of isotope.

Cytolysis of macrophages which had not been incubated with antigen was termed non-specific cytotoxicity. Cytolysis of macrophages incubated with PPD was termed antigen specific cytotoxicity.

MONOCLONAL ANTIBODY BLOCKING

The MHC restriction of PPD stimulated cells was determined by using antibodies directed against MHC class I (W6/32, A, B, C specific W6/32, SeraLab, UK) and class II (BU26, DP, DQ, DR specific, Dr M Goodall, Department of Immunology, Birmingham University) antigens. The antibodies were preincubated with target macrophages for 30 minutes before the addition of the effector cells and were present throughout the assay.

LYMPHOCYTE PROLIFERATION ASSAYS

Lymphocytes were isolated as described previously and added in triplicate to U bottom microtitre plates at a concentration of 1×10^5 well.[21 22] Antigen was added at the concentrations indicated previously and the plates were incubated for six days before the addition of tritiated thymidine (Amersham, UK) at 0·15 μCi/well for 16 hours. Cells were harvested using an automatic cell harvester (Skatron, Finland) by suction through glass fibre paper and radioactivity was assessed using a beta counter (LKB Rackbeta 2).

The stimulatory capacity of the antigen is expressed as a lymphocyte proliferation index and is calculated as follows:

$$\frac{\text{(mean dpm in cultures with antigen} - \text{mean dpm in cultures without antigen)}}{\text{mean dpm cultures without antigen}}$$

DEPLETION OF T CELL SUBSETS

Effector cells stimulated with PPD for seven days were generated from Mantoux positive control subjects as described above. These were used to obtain effector cells which were depleted of $CD4^+$ or $CD8^+$ lymphocytes. This depletion was performed by incubation with mouse monoclonal CD4 or CD8 antibodies (described in the surface marker section above) before a second incubation with goat anti-mouse immunoglobulin coated magnetic beads (Dynabeads, Dynal, Trondheim, Norway). For comparison, effector cells were used without any depletion procedure ("unmanipulated effector cells") or after sham depletion by incubation with anti-mouse immunoglobulin coated Dynabeads alone.

MAGNETIC SEPARATION AND CYTOTOXICITY ASSAY

Suspensions of effector cells and beads at a 20:1 bead target cell ratio were incubated for 90 minutes at 4°C in a glass test tube. Suspensions were then adjusted to 2 ml with phosphate buffered saline and placed next to a cobalt-sumarium magnet (Dynal, MPC-1) for two to three minutes. Dynabeads, including those bound to cells, adhered to the inside wall of the tube adjacent to the magnet. The unbound cell suspension was removed by careful aspiration into another tube. Depleted cells were pelleted by centrifugation for five minutes at 700 *g*, washed once with RPMI containing 10% autologous serum, and resuspended to 3×10^6/ml in RPMI.

The phenotype of the various populations was assessed using antibody coated sheep erythrocytes, before and after the depletion procedure, to ensure that $CD4^+$ or $CD8^+$ cells had been adequately removed. The above procedure normally resulted in >90% reduction of either CD4 or CD8 cells. The lysis mediated by unmanipulated, sham depleted, CD4 or CD8 depleted populations was tested using the cytotoxicity assay detailed above.

STATISTICAL ANALYSIS

The non-parametric Wilcoxon test was used for statistical analysis throughout. Measurements of statistical significance refer to comparisons made using an effector:target ratio of 20:1 unless otherwise indicated. Other tests used are quoted in the relevant results sections. Where ranges are quoted they correspond to maximum and minimum values.

Results

GENERATION OF CYTOLYTIC T CELLS BY CONTROL SUBJECTS AND PATIENTS WITH TUBERCULOSIS

Peripheral blood lymphocytes from the 32 normal donors and 36 patients with tuberculosis, when stimulated in vitro with 10 μg/ml PPD for seven days, lysed autologous macrophage targets in a dose dependent manner (figure 1 and table 1; individual values available from the author on request). There was a wide variation in the ability of lymphocytes to generate lymphoblasts capable of killing autologous macrophages in both groups. Macro-

Table 1 Cytolysis of autologous macrophages produced by purifed protein derivative (PPD) stimulated effector cells from patients with tuberculosis or normal control subjects (median (range))

	Target antigen/E:T ratio					
	No Ag		*SK/SD*		*PPD*	
Subject details	*20:1*	*5:1*	*20:1*	*5:1*	*20:1*	*5:1*
Diffuse non-cavitating/miliary (patient Nos 1–9)	10 (2–28)	3 (0–12)	20 (6–30)	8 (3–11)	27 (14–41)	13 (5–39)
Cavitating pulmonary/lymph node (patient Nos 10–36)	18 (5–39)	8 (0–36)	20 (10–40)	9 (0–30)	46 (35–62)	36 (19–47)
Normal controls (Mantoux negative, n = 8)	12 (0–15)	4 (0–14)	13 (7–13)	4 (3–7)	22 (15–46)	10 (4–24)
Normal controls (Mantoux positive, n = 25)	17 (5–49)	8 (1–34)	19 (5–49)	8 (4–36)	49 (28–60)	33 (18–52)

E:T refers to the effector:target ratio. SK = streptokinase, SD = streptodornase.

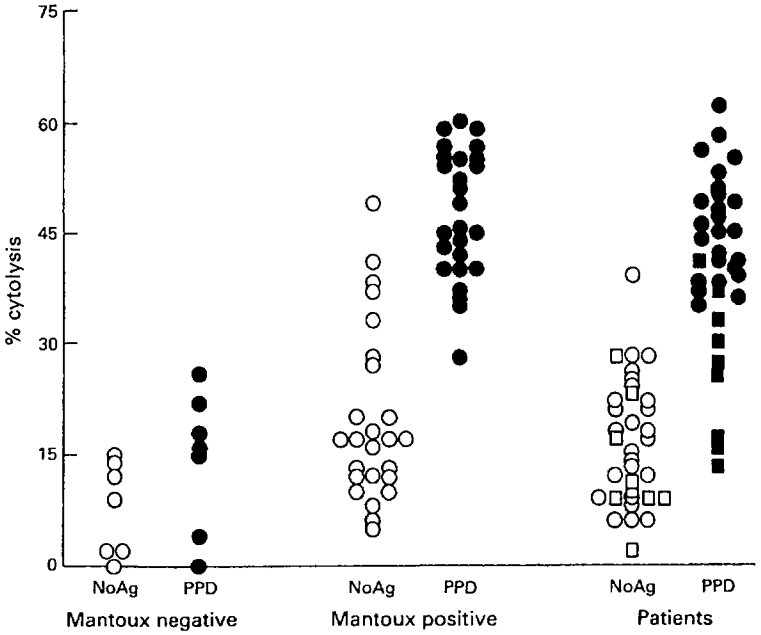

Figure 1 Percentage cytolysis of antigen unpulsed (open symbols) or purified protein derivative (PPD) pulsed (closed symbols) macrophages by PPD stimulated effector cells from Mantoux positive or Mantoux negative control subjects. Patients with diffuse non-cavitating or miliary tuberculosis are shown as squares. The effector:target cell ratio was 20:1.

phages pulsed with PPD were killed to a greater extent than those not pulsed with PPD (unpulsed) or macrophages pulsed with the control antigen streptokinase/streptodornase ($p < 0.001$ for all comparisons) in both groups.

PPD stimulated lymphocytes from normal BCG vaccinated subjects showed greater antigen specific cytolytic capacity than those from normal unvaccinated individuals ($p < 0.001$). PPD stimulated lymphocytes from patients with tuberculosis were less able to lyse PPD pulsed macrophages than those from normal BCG vaccinated subjects ($p < 0.03$). When patients were stratified according to clinical features, those with cavitating pulmonary disease and extrathoracic lymphadenopathy tended to generate higher amounts of antigen specific cytotoxicity than those with non-cavitatory pulmonary disease ($p < 0.001$). The cytolytic activity of PPD stimulated lymphocytes from patients with cavitatory disease did not differ significantly from those of BCG vaccinated control subjects ($p = 0.67$).

Normal subjects and patients showed similar levels of non-specific lysis (lysis of antigen unpulsed macrophages), which ranged from 10 to 90% of the total lysis in both groups. Lysis of macrophages pulsed with control antigen (streptokinase/streptodornase) did not differ from that seen with unpulsed macrophages in either group. No relation was found between the ability to generate antigen specific or non-specific cytotoxic T cells and age, sex, or race.

Interleukin 2 stimulated cells from Mantoux positive control subjects were only poorly cytotoxic to autologous antigen pulsed and unpulsed macrophages (figure 2), even though there was a good proliferative response to this cytokine (lymphocyte proliferation index >80).

Proliferative responses to mycobacterial antigen were measured in 22 normal subjects and in 11 patients. There was a weak positive correlation between lymphocyte proliferation and cytolysis of antigen pulsed macrophages ($r = 0.37$, $p > 0.04$) (figure 3).

LYMPHOCYTE SURFACE MARKER ANALYSIS

The surface phenotype of cells stimulated by PPD for seven days was characterised in parallel with cytotoxicity assays in 12 Mantoux positive control subjects and nine patients (table 2). The lymphoblast population in all groups consisted predominantly of $CD3^+$ cells, most of which were $CD4^+$ (table 2). Variable expression of HNK1 and CD11b (markers for natural killer cells) was found (data not shown). Only a minority of cells expressed the gamma/delta T cell receptor. There was no significant difference in the phenotype of cells from patients and control subjects and no statistical correlation between the CD4:CD8 ratio in the PPD stimulated cells and the level of specific or non-specific cytotoxicity.

Surface marker analysis of unstimulated lymphocytes from tuberculous pleural effusions from two patients also showed a predominance of $CD3^+$ and $CD4^+$ cells and low numbers of gamma/delta cells.

EVIDENCE THAT MYCOBACTERIAL ANTIGEN SPECIFIC LYSIS OF MACROPHAGES IS MHC CLASS II RESTRICTED

Antibodies specific for class II MHC antigens inhibited antigen specific lysis in both patients and normal control subjects (table 3). The mean inhibition observed at an antibody dilu-

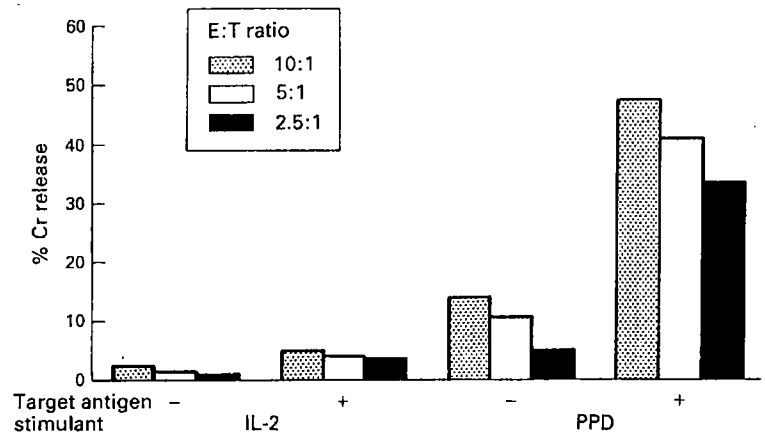

Figure 2 The lysis of autologous macrophages by peripheral blood lymphocytes stimulated in vitro with recombinant interleukin 2 (IL-2) (20 U) or with purified protein derivative (PPD) for seven days. Data pooled from two experiments using normal donors. E:T = effector:target ratio.

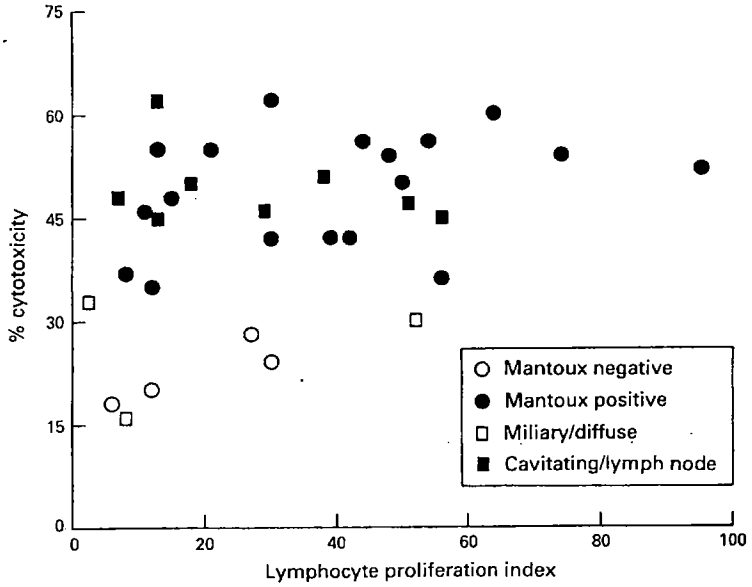

Figure 3 Relation of lymphocyte proliferation to purified protein derivative (PPD) (10 μg/ml) and the ability of these effector cells to kill PPD pulsed, autologous macrophage targets. Lymphocyte proliferation index is plotted against cytolysis of PPD pulsed targets at an effector:target ratio of 20:1.

tion of 1:400 was 45% ($p < 0.01$) in patients and 65% ($p < 0.001$) in control subjects. In contrast, no significant blocking or a modest enhancement of cytolysis was observed when antibodies directed against class I determinants were used.

T CELL SUBSET DEPLETION

PPD stimulated lymphocytes were depleted of $CD4^+$ or $CD8^+$ cells (as described in methods) and the depleted cell fraction was resuspended to give the final effector:target ratios indicated. Data pooled from four experiments are shown in figure 4. These experiments used cells from normal donors since only a limited number of peripheral blood cells could be obtained from patients. Effector cells which were treated with magnetic beads alone (without antibody treatment beforehand) were capable of lysing antigen pulsed or unpulsed target cells to the same extent as the unmanipulated fraction; thus, use of magnetic beads alone did not itself effect cytolysis.

Depletion of $CD4^+$ cells from PPD stimulated lymphocytes resulted in a decrease in antigen specific lysis of macrophages compared with unmanipulated or sham depleted fraction ($p < 0.001$ in each case). The residual killing of antigen pulsed targets by CD4 depleted cells was equivalent to that of unpulsed targets ($p = 0.940$; figure 4).

The effector cell fraction, which was depleted of CD8 bearing cells (and was therefore enriched for $CD4^+$ cells), showed an enhanced ability to kill mycobacterial antigen bearing macrophage targets ($p < 0.02$).

Discussion

We have shown that the generation of sensitised $CD4^+$ T cells can lyse autologous macrophages presenting mycobacterial antigen, and is thus an integral part of the immune response to both BCG vaccination and infection with *M tuberculosis*. This extends previous observations by ourselves and others.[16 17 20] Although the patients with tuberculosis had significantly less ability to generate these cytolytic T cells than did BCG vaccinated subjects, the difference was a result of the relatively poor

Table 2 Surface marker analysis of purifed protein derivative (PPD) stimulated lymphoblasts and unstimulated pleural fluid populations: individual patient data and control group data

	Surface marker (% of cells)			
Subject details	*CD3*	*CD4*	*CD8*	*γ/δ TCR*
Normal controls (median (range)) (n = 12)	82 (68–98)	48 (19–80)	49 (27–77)	5 (2–13)
Cavitating pulmonary/lymph node	90	69	14	6
Cavitating pulmonary/lymph node	88	82	7	3
Cavitating pulmonary/lymph node	92	82	17	2
Cavitating pulmonary/lymph node	77	46	26	nd
Cavitating pulmonary/lymph node	95	31	40	nd
Diffuse non-cavitating/miliary	86	38	50	nd
Diffuse non-cavitating/miliary	72	50	30	nd
Pleural fluid	48	53	18	2
Pleural fluid	93	69	19	3
Patient summary (median (range))	88 (48–95)	53 (31–82)	19 (7–50)	3 (2–6)

TCR = T cell receptor, nd = not determined.

Table 3 Effects of the addition of monoclonal anti-MHC class I or class II antibodies on the ability of purifed protein derivative (PPD) stimulated effector cells to kill autologous macrophage targets. (Results are shown as mean (SEM) lysis values)

		Antigen pulsed				
			Anti class I		Anti class II	
Subjects	No antigen, no antibody	No antibody	1:200	1:2000	1:400	1:4000
Patients (n = 7)	17·4 (3·3)	41·1 (3·3)	48·0 (2·9)	45·0 (2·9)	30·6 (3·8)	31·5 (4)
Controls (n = 7)	15·0 (2·8)	45·7 (4·7)	51·7 (5·6)	53·2 (5·1)	25·7 (4·1)	37·1 (5·8)

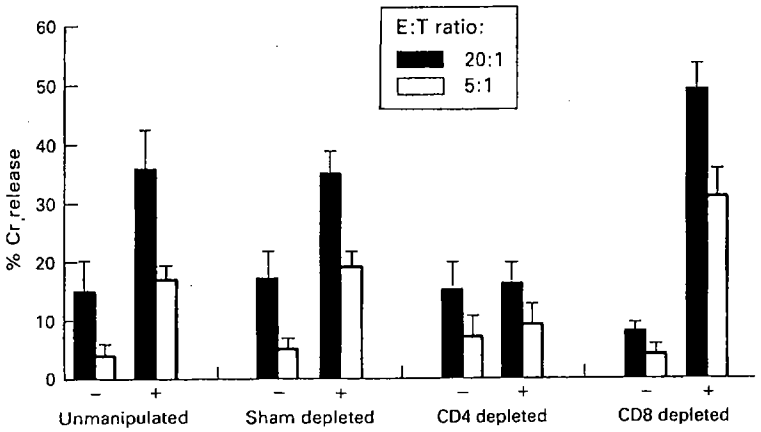

Figure 4 The effect of depleting CD4⁺ or CD8⁺ lymphoblasts respectively, on the ability of (PPD) stimulated effector cells to kill autologous macrophage targets. Mean (SEM) percentage lysis of PPD pulsed (+) or unpulsed (–) macrophages from four experiments, using Mantoux positive control subjects are shown. The effector populations were: PPD stimulated lymphoblasts used without further manipulation (unmanipulated); sham depleted by incubation with magnetic beads alone; CD4⁺ cell depleted, or CD8⁺ cell depleted.

cytolytic activity of PPD stimulated lymphocytes from patients with non-cavitatory pulmonary disease. In contrast, patients with cavitatory disease and those with extrathoracic lymph node disease had an ability to generate cytolytic T cells similar to that of BCG vaccinated subjects. Although only a small number of patients were studied, those with miliary disease seemed to have only a moderate capacity to generate cytolytic T cells.

Approximately 20% of patients with extensive pulmonary tuberculosis and a greater proportion of those with miliary disease are anergic as shown by absent cutaneous tuberculin hypersensitivity and poor in vitro lymphocyte proliferative responses to mycobacterial antigen.[23 24] Most of the patients with poor cytolytic T cell activity described here also showed relatively poor in vitro lymphocyte proliferation (and absent cutaneous hypersensitivity), and were thus anergic. There was, however, only a poor correlation between cytolytic T cell activity and lymphocyte proliferation overall. This finding contrasts with that of Kaleab *et al*,[18] who found a close correlation between cytolytic T cell activity and lymphocyte proliferation in patients with leprosy and healthy control subjects. Whole BCG was used to stimulate effector cells in their study, however, while we used PPD. Furthermore, we observed that mononuclear cell populations from several of our patients showed spontaneous proliferation in the absence of antigen and this can give a falsely low lymphocyte proliferation index. Other evidence suggests that proliferation in response to antigen and antigen specific cytotoxicity are essentially unrelated T cell functions.[25] The data presented here show that Interleukin 2 stimulated T cells, which show good proliferative responses, are poorly cytolytic.

We have confirmed previous data[18 20 21] showing that the cytolytic activity of PPD stimulated lymphocyte populations has both antigen specific and antigen non-specific components. We have also shown that in addition to the considerable variation in total cytolytic T cell activity between individuals, the relative contribution of antigen specific and antigen non-specific components to the total cytolysis varies considerably between individuals, whether patients or control subjects.

From previous studies of BCG vaccinated subjects and patients with leprosy and tuberculosis there is good evidence that CD4⁺ cells restricted by MHC class II antigens are responsible for the cytolysis of antigen presenting macrophages.[16–18 20 21] This view is supported by our finding that depletion of CD4⁺ but not CD8⁺ cells removed the antigen specific component of cytotoxicity. Cytolysis of antigen unpulsed macrophages was not significantly affected by CD4⁺/CD8⁺ depletion. Antigen non-specific cytolysis, however, is inhibited by coculture of macrophages with K562 cells as competing target cells, suggesting that cells with natural killer like activity are responsible.[20] An alternative suggestion is that gamma/delta T cell receptor bearing lymphocytes may be responsible for antigen non-specific cytolysis.[26] Gamma/delta T cells show broad cytolytic activity after culture with interleukin 2,[27] proliferate to mycobacterial antigens in both MHC restricted and unrestricted manner,[28 29] and have been identified in granulomatous lesions of human leprosy and leishmaniasis.[30] In this study, however, gamma/delta cells accounted for a small proportion only of the PPD stimulated populations and are therefore unlikely to have contributed greatly to the antigen specific or non-specific cytolysis shown. Furthermore, we observed that lymphocyte populations from tuberculous pleural fluid that had not been restimulated in vitro, showed low numbers only of gamma/delta cells.

There has been considerable speculation recently about the role of cytolytic T cells in the immune response to tuberculosis.[26] This results largely from the failure to achieve effective mycobacteriostasis with lymphokine activated human macrophages in vitro.[9 31] In

addition murine cytolytic T cells can inhibit mycobacterial growth independently of lymphokine activated macrophages.[14] Furthermore, both adoptive transfer and selective depletion experiments in mice have shown that CD8+ cytolytic T cells provide protection against *M tuberculosis* infection in vivo.[15 32] Mononuclear cells in tuberculous granulomata show functional heterogeneity[33] and tissue macrophages seem to possess significantly less antibacterial capacity than blood monocytes.[34] It has been suggested consequently that cytolytic T cells may have a pivotal role in antimycobacterial immunity by releasing mycobacteria from the safe haven of poorly activated tissue macrophages so that they can be phagocytosed by the more active, blood derived monocytes. Extracellular mycobacteria would also be exposed to toxic macrophage products released from dying macrophages. Some support for this hypothesis is provided by the observation that reconstitution of cellular immunity in lepromatous leprosy lesions by intralesional interleukin 2 is associated with infiltration of CD4+ and CD8+ cells. There is subsequent lysis of parasitised macrophages within these lesions followed by rephagocytosis of the released bacilli by freshly migrated monocytes and a concomitant reduction in the bacterial count.[19]

The finding of high levels of mycobacterial-antigen specific, cytolytic T cells in certain groups of patients with tuberculosis strongly suggests that the ability to generate these cells does not equate with protective immunity. There was, however, a strong association of cytolytic activity and tissue destruction. All patients with pulmonary cavitation could generate significant amounts of cytolytic activity and high levels were also found in those with tuberculous lymphadenitis, many of whom formed cold abscesses that required open or closed drainage. It is notable that patients with pneumonic tuberculosis and miliary disease had a reduced capacity to generate mycobacterial-antigen specific, cytolytic T cells. We therefore suggest that in tuberculosis cytolytic T cells may be involved in delayed type hypersentivity and contribute to tissue destruction.

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